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**REVIEW OF MODIFIED HEMOGLOBIN SOLUTIONS
FOR USE AS TEMPORARY BLOOD SUBSTITUTES**

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INTRODUCTION

Blood is the common carrier of nutrients, oxygen and metabolic products within the body. The loss of this carrier and the transport system for more than a few minutes results in irreversible damage to vital organs, and death. Blood also functions in the regulation of the acid-base balance and in immunologic reactions. The complex nature of blood, the composition of which is determined by and is unique to each individual, precludes the development of a true blood substitute. Temporary blood substitutes, which have an oxygen-transport capacity comparable to blood, however, have been shown to be capable of sustaining life following massive blood replacement (1-3, and references therein). The two types of compounds having sufficient oxygen-carrying capacities to be useful as blood substitutes include solutions of hemoglobin and fluorocarbon emulsions, although, in the case of the fluorocarbon emulsions, this capacity is achieved only when ventilation is performed with air containing 50-100% oxygen (3-6).

Conventional blood is probably the ideal resuscitative fluid under most normal circumstances. It has, however, several limitations such as a short storage life of about 35 days, and its supplies are limited. These limitations are especially significant in combat situations where compatible whole blood is often unavailable for the treatment of trauma victims involving massive hemorrhage (7). It also requires specialized pre-transfusion handling such as typing, tests for pathogens and cross-matching. In addition, in a small percentage of cases, complications arise from whole blood transfusions such as infection with blood borne pathogens, hemolytic transfusion reactions and allergic reactions. An oxygen transporting fluid which has a long storage life, is non-antigenic and which is pathogen free could supplement the blood banking services. It would be particularly useful in emergency situations where supplies of compatible blood are not available and its use during operations requiring large amounts of blood would allow for a more efficient use of whole blood.

There has been a continuing interest in the evaluation of fluids as blood substitutes since the late eighteen hundreds (8). Significant progress towards the development of blood substitutes capable of sustaining life however, came only after the preparation of suitable oxygen carriers in the late nineteen-sixties. In 1967, Rabiner and coworkers (9) showed that many of the toxic effects associated with the use of hemoglobin solutions as oxygen carriers could be eliminated by the use of stroma-free hemoglobin. Such a solution was used to replace the entire blood of rats and by continuous infusion, was shown to be capable of sustaining the life of rats until they regenerated their blood cells and plasma proteins (10, and references therein). About the same time as Rabiner and coworkers performed their work with hemoglobin, Clark and Gollan (11) described their liquid breathing experiments in which they showed that mice could survive for extended periods fully submerged in a solution of perfluorocarbon which was saturated with oxygen. Two years after Clark and Gollan's demonstration, Geyer and coworkers (12) showed that rats could live with their entire blood replaced with fluorocarbon emulsions.

These demonstrations of the effectiveness of hemoglobin and fluorocarbon emulsions as oxygen-carriers capable of sustaining life were followed by increased interest and rapid progress in the development of blood substitutes based on these carriers. In the case of the fluorocarbon emulsions, clinical testing began in the late 1970's, or about a decade after their first use in animals (13,29,33-35). However, in the opinions of some workers in the blood substitute field, the fluorocarbons had been pushed to clinical testing before their time (4). In the case of the hemoglobin solutions, progress has been slower. In the first clinical testing of stroma-free hemoglobin which was prepared by a modification of the procedure described by Rabiner *et al.* (9), Savitsky *et al.* (14) observed various toxic reactions. These included a mild prolongation of the activated partial thromboplastin time, bradycardia, a mild increase in blood pressure and a decrease in urine output and creatinine clearance. Recent studies with dogs, pigs and monkeys have shown that coagulation changes were absent in pigs and monkeys when the animals were infused with lipid-poor hemoglobin solutions, although dogs still exhibited significant transient thrombocytopenia (15). In addition to the toxic effects, some of which may be associated with contaminants in the stroma-free hemoglobin preparations, the rate of progress has been slower with the hemoglobin solutions due to their short intravascular half-life of 2-4 hours and the high oxygen affinity of extracellular hemoglobin (16). The purification procedures and the chemical modification approaches taken to overcome these limitations of hemoglobin solutions will be discussed below.

The progress made towards the development of blood substitutes using hemoglobin and fluorocarbon emulsions has been discussed in several recent reviews (17-25). Both types of blood substitutes offer certain advantages as well as have certain limitations. These have been discussed along with comments on their potential uses (26). An important limitation of the fluorocarbon emulsions which limits their use under certain emergency situations is the need to give 50-100% oxygen to the individual receiving the fluorocarbon-based substitute. Hemoglobin solutions, on the other hand, are effective oxygen-carriers at ambient oxygen tensions (24). In addition, hemoglobin is a physiologically suitable oxygen carrier capable of unloading its oxygen within a narrow range of oxygen tensions as a result of the cooperative effects between the subunits in the hemoglobin tetramer (53). The perfluorocarbon emulsions release their oxygen in a linear fashion and thus require comparably large changes in oxygen tensions to release the same amount of oxygen as hemoglobin (see Figure 1 in Reference 6). Also, hemoglobin is oncologically active, whereas the fluorocarbon emulsions require the addition of oncologically active agents such as hydroxyethyl starch (3 percent) to be effective (4).

The present review will focus on blood substitutes using hemoglobin solutions as oxygen carriers. It will review the more recent developments and discuss the limitations and problems associated with the present hemoglobin preparations. This will be covered under the headings of purification of hemoglobin, modification and polymerization of the hemoglobin molecule and the biological activities of the hemoglobin solutions.

PURIFICATION OF HEMOGLOBIN

The early hemoglobin solutions (27, and references therein) were found to be unsuitable as blood substitutes due to their toxic effects (8) and, in particular, their effects on renal function (28). The demonstration by Rabiner *et al.* (9) that many of the toxic effects of hemoglobin solutions could be eliminated by the use of stroma-free hemoglobin preparations has not only renewed interest in the use of hemoglobin as a blood substitute, but has also emphasized the need for proper purification of hemoglobin solutions. The various early procedures for the preparation of hemoglobin solutions have been reviewed by DeVenuto (18). There are currently two general approaches for large-scale purification of hemoglobin. One is based on crystallization of hemoglobin in the presence of high salt concentrations, and the other on tangential-flow ultrafiltration.

Crystallization of hemoglobin has been described as early as the 1840's (see bibliography in Reference 30); however, a large-scale and an efficient purification process based on crystallization has been described only relatively recently by Drabkin (31) which was further modified in 1977 by DeVenuto *et al.* (32). This procedure is summarized in Figure 1. In this procedure, washed red blood cells are lysed in 1 volume of distilled water and 0.4 volumes of toluene. Particulate matter, which includes the red blood cell membranes, is removed by filtration through a surgical gauze followed by centrifugation at 4000 g. The hemoglobin is then crystallized by dialysis against 2.8 M phosphate buffer. The hemoglobin crystals are washed and dissolved in distilled water. After dialysis against a desired buffer the hemoglobin solution is sterilized by filtration through a 0.22 micron Millipore filter. This process offers the possibility of excellent yields of hemoglobin as practically all of the pigment within the dialysis sac is obtained in the crystalline form (31). The advantage of this approach over that used by Rabiner *et al.* (9) is that it eliminates the need for high speed centrifugation and allows for more complete removal of the stromal components such as soluble stromal lipids (14,15). The hemoglobin prepared by the crystallization procedure was shown to be free of type-specific erythrocyte antigens, however, it is still contaminated with carbonic anhydrase (32), as well as by small amounts of the red cell glycolytic enzymes (see page 425 in Reference 18). The methemoglobin levels in these hemoglobin solutions range between 2.2 and 4.1 percent.

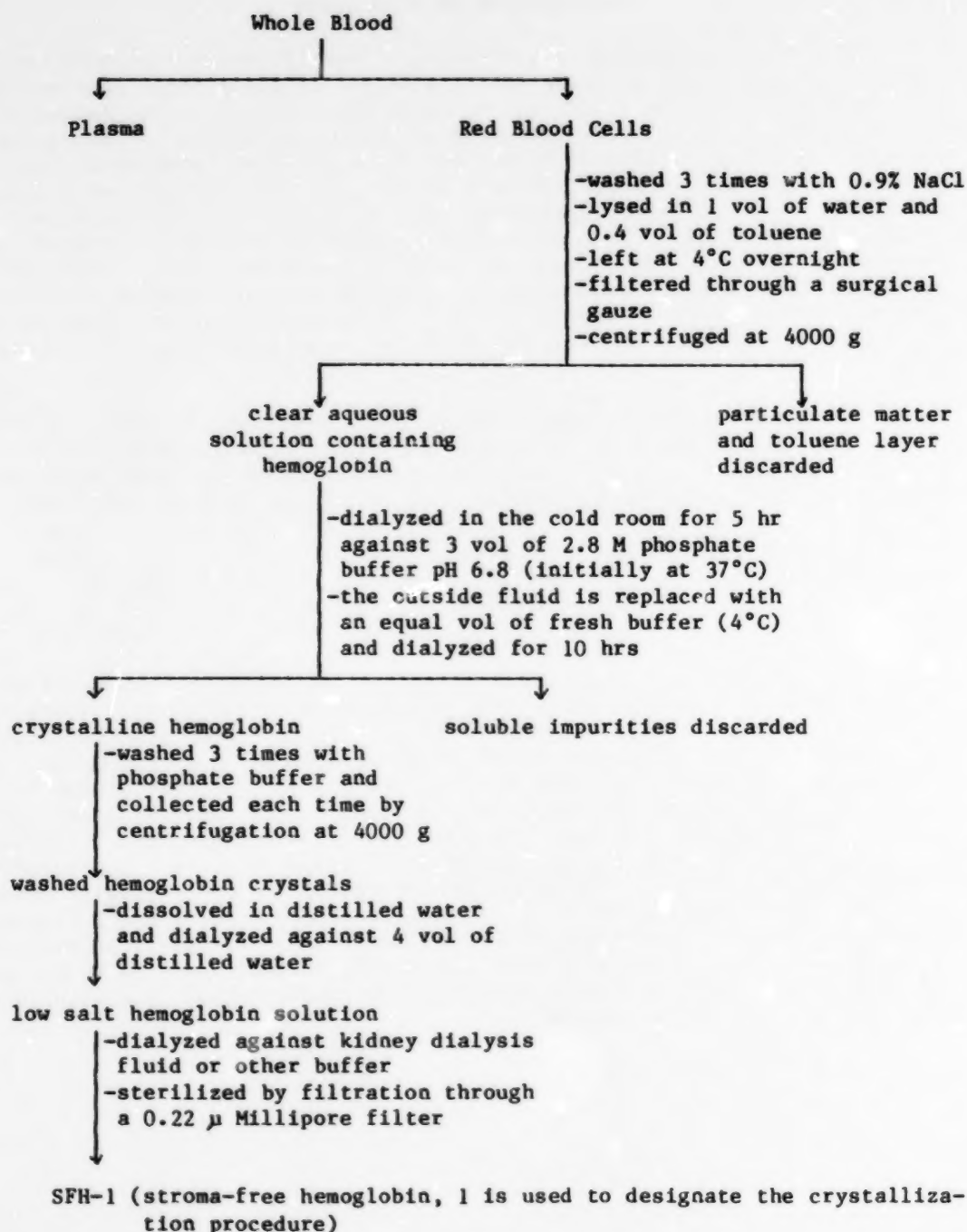


Figure 1. Procedure for the preparation of stroma-free hemoglobin by the crystallization approach

The tangential-flow ultrafiltration approach has been described by several groups (36-39) and has been applied at the University of Minnesota for the large scale production of stroma-free hemoglobin (38). Figure 2 summarizes the steps in this approach. Centrifugation in this approach is used only in the first step for the separation of the red blood cells from plasma. The washed cells are lysed in a hypotonic phosphate buffer (20 mOsm) (41). The hemolysate is then filtered through a series of ultrafiltration membranes using a tangential flow membrane filtration system. In the first series of filtrations, the hemoglobin and soluble cellular components are separated from the red blood cell membranes by filtration through a 100,000 daltons molecular weight cut-off filter. The hemoglobin solution is then concentrated and separated from small molecules by filtration through a membrane with a molecular weight cut-off of approximately 10,000 daltons. The solution is then dialyzed against an appropriate buffer and sterilized by filtration through a 0.22 micron Millipore filter. The hemoglobin solutions prepared by this approach are still contaminated by the other cytoplasmic proteins found in the red cell. Hedlund *et al.* (38) showed that such hemoglobin preparations still contained 70-80% of the superoxide dismutase activity found in the red cells. These solutions, however, were shown to be free of phospholipids such as phosphatidylcholine and sphingomyelin (36). The methemoglobin of these solutions is between 1 and 2 percent (36,39). Tye and coworkers (42) have described a procedure for removal of non-heme proteins by selective precipitation of the zinc-hemoglobin complex.

The characterization of the hemoglobin for purity and for heterogeneity may be performed by a variety of column chromatographic techniques (46, and references therein). Characterization by cellulose acetate electrophoresis has been performed according to the procedure described by Lehmann and Huntsman (47). The separation of the different hemoglobins has been described previously (48,49). The measurement of the various parameters of the hemoglobin solutions, such as the concentration, percent oxyhemoglobin, carboxyhemoglobin and methemoglobin, may be made on an IL 282 Cooximeter as described previously (50,51). The methemoglobin content can also be determined by fractionation of the hemoglobin solution using isoelectric focusing on polyacrylamide gels (60,61). Sterility (32) and gross toxicity tests (1) may be performed as described previously. Information on the hemoglobin's affinity for oxygen has been obtained by analysis of the hemoglobin solution utilizing the Hem-O-Scan (52). The above series of analyses provide information not only on the purity and heterogeneity of the hemoglobin solutions, but also on their physiological integrity.

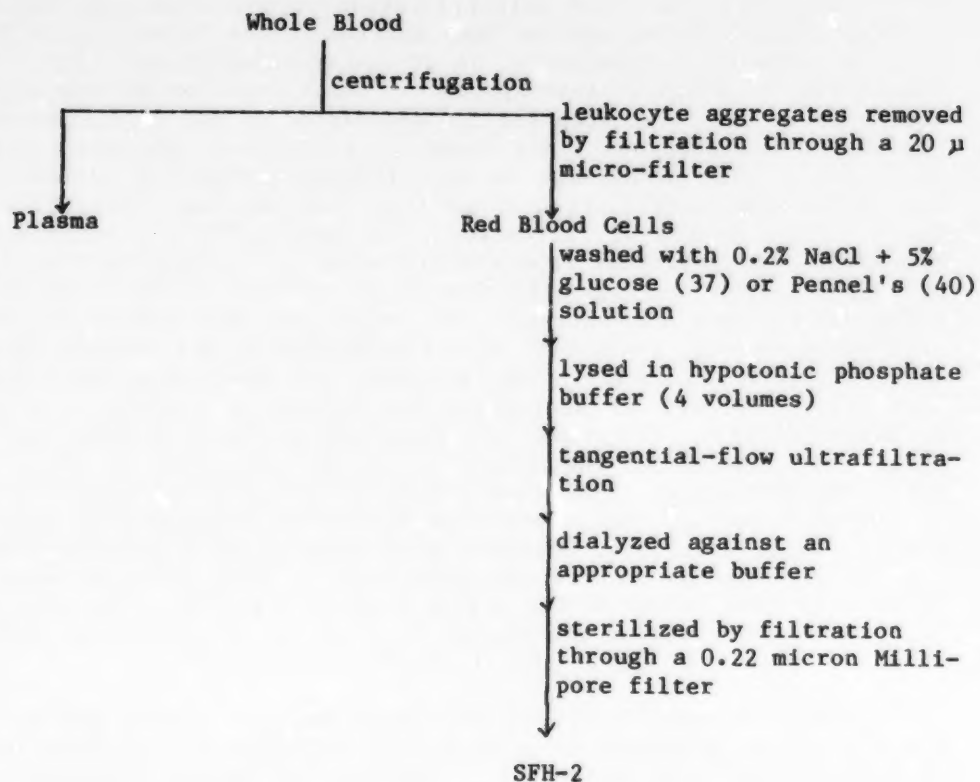


Figure 2. Preparation of stroma-free hemoglobin by the ultrafiltration approach.

MODIFICATION OF HEMOGLOBIN

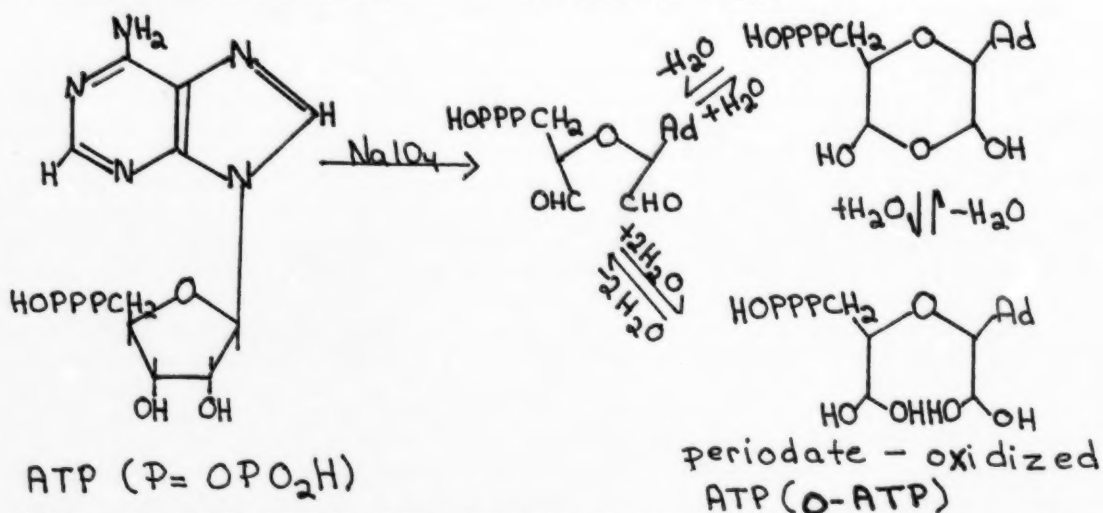
Purified hemoglobin solutions have several serious deficiencies which limit their usefulness as blood substitutes. These deficiencies, as discussed below, include high oxygen affinity, short intravascular retention time, and high oncotic pressure.

The oxygen-binding characteristics of hemoglobin are highly dependent on its environment (55). Hemoglobin is an allosteric protein and its affinity for oxygen is profoundly affected by its interaction with organic phosphates, such as 2,3-diphosphoglycerate and ATP (54). These modulators, which interact with hemoglobin in a reversible fashion, are lost when the hemoglobin is removed from the red blood cell. In the absence of these modulators, hemoglobin has a much higher affinity for oxygen as indicated by P_{50} values of 26-27 and 12-16 torr for intraerythrocytic and purified hemoglobins, respectively (23). In addition, extraerythrocytic hemoglobin has a short intravascular half-life of about 2-4 hr (23). This rapid loss from the vascular system occurs as a result of the dissociation of the tetrameric molecule into dimers (56) which then bind to serum haptoglobin. The complexes are then rapidly removed from the circulation by the reticuloendothelial system with a disappearance half-time of 9-30 min (57). When the plasma hemoglobin concentration exceeds the binding capacity of haptoglobin, the same dissociation into the lower molecular weight dimers allows the hemoglobin to filter through the glomeruli. Reabsorption of hemoglobin occurs in the proximal tubules, however, when the concentration exceeds the reabsorption capacity of the proximal tubular cells it is excreted by the kidneys (18,58). The third important consequence resulting from the removal of hemoglobin from the red blood cell, which limits its usefulness as a blood substitute, concerns the oncotic activity of hemoglobin solutions. While it is essential that a blood substitute is oncologically active to prevent circulatory collapse, a solution of hemoglobin which has the same concentration of hemoglobin as whole blood has an unacceptable oncotic pressure of 65 torr, which is more than three-times that due to plasma proteins (19 torr) (59).

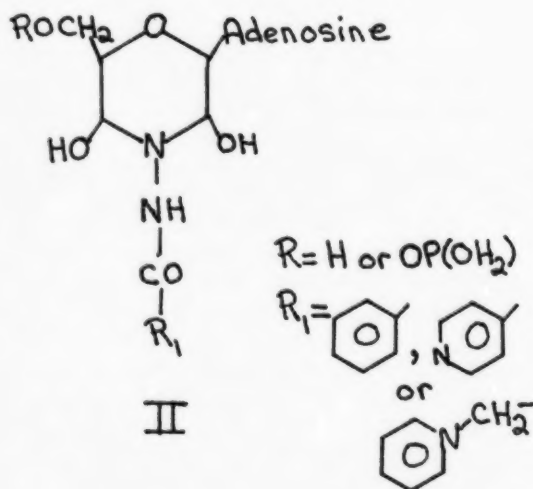
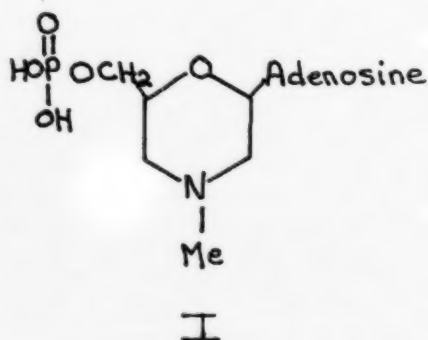
In order to overcome the above deficiencies of purified hemoglobin solutions, various chemical modifications of hemoglobin have been developed. These modifications include covalently linking modifiers in the hemoglobin's phosphate binding pocket in order to decrease its oxygen affinities to those found in the red blood cells. The problem of short intravascular half-life and high oncotic pressure has been addressed by various chemical modifications which include intramolecular cross-linking to stabilize the tetramer, tetramer-tetramer cross-linking or polymerization, and covalently linking the hemoglobin to other polymers in order to increase its molecular weight. The various modification techniques have been reviewed by DeVenuto (23) in 1983. The present review will focus on the more recent work and on the techniques currently used for hemoglobin modification.

The modifiers that are currently used for lowering the oxygen affinity of hemoglobin (i.e., raising the P_{50} value) include pyridoxal phosphate (PLP) (54,62,78,83), glyoxylate (63-67), glucose 6-phosphate

(71,72), periodate-oxidized nucleoside triphosphates (o-NTP) (68,69), periodate-oxidized 5-phosphorylribose 1-pyrophosphate (o-PRPP) (68,70) and 2-nor-2-formylpyridoxal 5'-phosphate (73-77). Each of the first three modifiers is linked to one of the chains in tetrameric hemoglobin by a stable covalent bond. In the case of the modifiers prepared by periodate oxidation of the nucleotides or the PRPP, the nature of the linkage to the hemoglobin molecule is less certain. These modifiers have potentially two aldehyde functional groups as shown below for o-ATP.



Thus, one might expect these reagents to serve as cross-linking modifiers as has been suggested previously (68,69). It has been shown, however, that treatment of periodate-oxidized adenosine 5'-phosphate (AMP) with methylamine and then with sodium borohydride leads to a morpholine derivative (I), in which both of the aldehyde carbons are bonded to the same amino group (80). Similar stable morpholine derivatives (II) have been obtained by reacting periodate-oxidized nucleosides and nucleotides with carboxylic acid hydrazides (81).



The o-PRPP-modified hemoglobin prepared by Maffuid et al. (70) was characterized by cellulose acetate electrophoresis, and the degree of cross-linking by SDS-polyacrylamide gel electrophoresis. These authors (70) state that o-PRPP-modified hemoglobin gave a single electrophoretic product and that cross-linking occurred to the extent of about 10%. In addition, they stated that o-ATP-modified hemoglobin was cross-linked to the extent of 60%, although the o-ATP-modified hemoglobin gave a more complex electrophoretic pattern and required complex purification (70). The observations made by Maffuid et al. (70), Greenburg et al. (69) and Scannon (68), on the cross-linking of hemoglobin by o-PRPP and the periodate-oxidized nucleotides, are particularly interesting in view of the nature of the reaction of periodate-oxidized nucleosides and nucleotides with primary amines and hydrazides (80,81). Additional studies are required, therefore, in order to elucidate the nature of the cross-linking observed by the above authors (68-70).

Benesch et al. (73) showed that the modifier, 2-nor-2-formylpyridoxal 5'-phosphate, not only profoundly lowers hemoglobin affinity for oxygen, but also crosslinks the hemoglobin tetramer between the beta-chains. The position of the crosslink was shown by X-ray crystallography to be between the N-terminal amino group of one beta-chain and lysine-82 of the other (74). The hemoglobin thus modified, is prevented from dissociating into alpha beta dimers and is not excreted in the urine, unlike the uncrosslinked pyridoxylated hemoglobin (76).

An important feature of all of the above modifiers is their anionic nature which allows them to interact with the 2,3-diphosphoglycerate (2,3-DPG) binding site and, like 2,3-DPG, all decrease hemoglobin's affinity for oxygen. A direct comparison of oxygen affinities of hemoglobins modified by the different modifiers is not possible since different laboratories have used different procedures for determining the P_{50} values. Each laboratory, however, has compared the oxygen affinity of modified hemoglobin with the oxygen affinity of unmodified hemoglobin. Thus, the effect of modification can be expressed in terms of the P_{50m}/P_{50um} ratio, where P_{50m} and P_{50um} characterize the oxygen affinities of the modified and unmodified hemoglobins, respectively.

Table 1 summarizes the oxygen affinities and the Hill coefficients for the various modified and unmodified hemoglobins. The oxygen affinities of the pyridoxal 5'-phosphate [$\alpha_2(\beta \text{ PLP})_2$], o-ATP, glucose 6-phosphate and o-PRPP modified hemoglobins are very similar as indicated by the P_{50m}/P_{50um} ratios of 2.8, 3.0, 3.1 and 3.4, respectively. The unpurified pyridoxylated stroma-free hemoglobin (SFH-P) shows a higher oxygen affinity (as indicated by ratios of 1.4-2.0) than the purified material (SFH-P purified), due to the presence of unmodified hemoglobin in the preparation. Purification with DEAE-Sephadex, which increased the percentage of pyridoxylated hemoglobin from 70 to 85 percent, gave a SFH-P product which had a P_{50} of 28 torr and an n of 2.0 (78). The affinities of the glyoxylated hemoglobins, as shown in Table 1, are highly dependent on the number and position of attachment of the glyoxylate groups. As shown in Table 1 by the P_{50} values of 7, 12, 17 and 37 torr for $\alpha_2\beta_2$, $\alpha_2^{cm}\beta_2$, $\alpha_2\beta_2^{cm}$ and $\alpha_2^{cm}\beta_2^{cm}$, respectively, modification of the beta-chains

produces a greater increase in the P_{50} than the modification of the alpha-chains and a much larger increase in P_{50} when all four N-terminal amino groups are modified. The modifier 2-nor-2-formylpyridoxal 5'-phosphate produces the largest decrease in hemoglobin's affinity for oxygen, as shown by a ratio of 7.1 (Table 1). This modifier provides a hemoglobin (HbXL) which has a significantly lower affinity for oxygen than blood and which can unload much more oxygen than blood (77). It has been suggested that the modifier 2-nor-2-formylpyridoxal 5'-phosphate would be ideal since it both provides a favourable oxygen affinity and crosslinks the tetramer, however, at the present it cannot be commercially synthesized (42).

TABLE 1
Oxygen affinities and Hill coefficients (n) of
modified and unmodified hemoglobins

Hemoglobin	P_{50} (Torr)		$\frac{P_{50m}}{P_{50um}}$	n	Reference
	unmodified P_{50um}	modified P_{50m}			
$\alpha_2\beta_2$	3.5			2.7	73
$\alpha_2(\beta^{PLP})_2$		10	2.8	2.6	82
HbXL		25	7.1	1.9	73
SFH	12-14			1.6-2.5	78
SFH-P		20-24	1.4-2.0	1.6-2.5	78
SFH-P purified		28	2.2	2.0	78
SFH	3.7			2.6	70
SFH-ATP		11	3.0	1.8	69,70
SFH-PRPP		12.5	3.4	1.7	70
HbA	3.5			2.8	72
G6PHb		11	3.1	2.3	72
$\alpha_2\beta_2$	7				64
$\alpha_2^{cm}\beta_2$		12	1.7		64
$\alpha_2\beta_2^{cm}$		17	2.4		64
$\alpha_2^{cm}\beta_2^{cm}$		37	5.3		64

Abbreviations: $\alpha_2\beta_2$, unmodified hemoglobin A; $\alpha_2(\beta^{PLP})_2$, hemoglobin modified with pyridoxal 5'-phosphate; HbXL, hemoglobin cross-linked with 2-nor-2-formylpyridoxal 5'-phosphate; SFH, stroma-free hemoglobin; SFH-ATP, stroma-free hemoglobin modified with periodate-oxidized adenosine triphosphate (o-ATP), SFH-PRPP, stroma-free hemoglobin modified with periodate-oxidized 5-phosphorylribose 1-pyrophosphate; $\alpha_2^{cm}\beta_2$, $\alpha_2\beta_2^{cm}$ and $\alpha_2^{cm}\beta_2^{cm}$, hemoglobin modified with glyoxylate at the terminal amino groups of the α , β and α and β chains respectively; SFH-P, pyridoxylated stroma-free hemoglobin; G6PHb, hemoglobin modified with glucose 6-phosphate.

All of the above modifiers have the capacity to provide a hemoglobin solution with the required oxygen affinity, however, in practice, the optimum affinities are difficult to achieve since modification is usually incomplete and, therefore, provides modified hemoglobins having less than optimum oxygen affinities. The optimum affinities are usually obtained after expensive purification procedures. In addition, most of the above modifiers do not cross-link the hemoglobin tetramer and the resulting modified hemoglobin solutions have relatively short intravascular retention-times. In the case of pyridoxylated hemoglobin, the intravascular retention time in dogs is only 1.5 times greater than that of the unmodified hemoglobin (84). The o-ATP and o-PRPP modified hemoglobins showed relatively longer retention times in the rat, with half-disappearance times of 202 ± 25 min (69) and 223 ± 49 min (70), respectively, compared to 106 ± 9 min (69) for unmodified hemoglobin.

The approach taken by Tye *et al.* (42) at improving the intravascular retention time of modified hemoglobin has been to first stabilize the hemoglobin tetramer by crosslinking with bis(3,5-dibromosalicyl)fumarate. This reagent reacts selectively with oxyhemoglobin to form a crosslink between lysine-82 of the beta-chains (85). Although the oxygen affinity of the above modified hemoglobin is similar to unmodified hemoglobin, a further modification with pyridoxal 5-phosphate provides a stabilized tetramer with a P_{50} of 32 torr and a significantly improved intravascular retention time. Preliminary results have shown a 20-hour half-disappearance time in the rabbit (42).

The technique most commonly used for increasing the intravascular time involves intermolecular crosslinking or polymerization of pyridoxylated (86-93), or glyoxylated (67) hemoglobin with glutaraldehyde. In addition to increasing the molecular weights of hemoglobin solutions, polymerization reduces their colloid osmotic pressure (COP) which is proportional to the number of colloid particules present. This allows the use of solutions having much higher hemoglobin concentrations which, for unpolymerized hemoglobin solutions, are limited to about 7% by their COP. The polymerization produces a heterogeneous polydispersed mixture of widely different molecular weights. The range of molecular weights is dependent on the degree of polymerization. An estimate of the molecular weights of a pyridoxylated polymerized hemoglobin (poly SFH-P) by gel filtration on a Sephadex G-200 column showed that in a 15% polymerized pyridoxylated hemoglobin solution, which is iso-oncotic with plasma, approximately 60% of the molecules have an apparent molecular weight of 600,000, 20% fall in the 120,000-600,000 range and about 20% are unpolymerized (89). An analysis of the above hemoglobin by SDS-PAGE for polypeptide molecular weight distribution showed 6-7 peaks having molecular weights in multiples of the hemoglobin monomer in the 17,000-112,000 dalton range.

The analysis and characteristics of the modified, cross-linked and/or the polymerized hemoglobin may be performed as described for the analysis of purified hemoglobin solutions. The presence of cross-linking may be determined by SDS acrylamide gel electrophoresis as described by Weber and Osborn (95). In addition, a variety of column chromatographic

techniques have been described for the characterization of modified and/or polymerized hemoglobin solutions (46, 96-99).

The most dramatic effect of polymerization is the increase in the intravascular half-life which ranges from 6-10 times that of SFH hemoglobin (1,86,90,92). DeVenuto and Zegma (90) have determined a plasma half-life of 25 hours in rats which had 93-95% of their blood replaced with pyridoxylated polymerized hemoglobin, compared to 3.5 hr for unmodified hemoglobin. Seghal *et al.* (86) observed a plasma half-disappearance time, of poly-SFH-P, in the baboon of 38 hours which was 10-fold greater than for unmodified hemoglobin. In addition, polymerized hemoglobin is not excreted in the urine as is the case with modified hemoglobin. The small amount of hemoglobin that is excreted in the urine of animals transfused with polymerized hemoglobin solutions, has been shown to consist of the unpolymerized hemoglobin fraction (1,92).

The major disadvantage of polymerization with glutaraldehyde is the decrease in the P_{50} value. The polymerized pyridoxylated hemoglobin prepared by Seghal *et al.* (89) has a P_{50} value of 14-16 torr which is practically the same as the P_{50} (12-14 torr) of unmodified hemoglobin solution (78). A higher P_{50} (19-22 torr) was obtained by DeVenuto and Zegma (90) for their pyridoxylated polymerized hemoglobin, however, this value is also lower than that of the pyridoxylated hemoglobin (23-26 torr). The other disadvantage of the polymerized hemoglobins is their relatively high methemoglobin content of 5-10%. The increased intravascular retention time and lower oncotic pressures are thus achieved at the expense of higher oxygen affinities and lower purities.

BIOLOGICAL ACTIVITIES OF HEMOGLOBIN SOLUTIONS

Many of the *in vivo* activities of hemoglobin solutions have been reviewed by DeVenuto in 1979 (18). However, since the publication of that review, blood substitutes based on hemoglobin have evolved from the stroma-free hemoglobin solutions to the modified and polymerized modified hemoglobin solutions. These modifications, in addition to improving the oxygen affinity characteristics and the intravascular retention time, may also alter other biological activities of the hemoglobin solutions. Thus, although most evidence shows that hemoglobin is nonantigenic or has very low antigenicity, the new modified and polymerized hemoglobin solutions will also have to be evaluated for antigenic reactions. The present review will focus on the biological activities of hemoglobin solutions which may limit their usefulness as blood substitutes. These biological activities include impairment of the coagulation activity, vasoactive effects, reticuloendothelial blockade and immunosuppression, oxygen unloading, iron deposition and nephrotoxicity.

The observations on the coagulation abnormalities produced by hemoglobin solutions are highly variable and depend on the experimental design, the animal system used and the composition of the hemoglobin solutions. Coagulation abnormalities resulting from the infusion of autologous hemolyzed erythrocytes have been observed in dogs (100) and primates (101,102), but not in rabbits (103) and man (104). Rabiner *et*

al. (9), however, observed no coagulant activity in dogs using hemoglobin solutions which were prepared by lysis of the erythrocytes with hypotonic phosphate buffer and high speed centrifugation, followed by micropore filtration to remove the RBC stroma. Savitsky et al. (14), using a hemoglobin solution prepared by a modification of the method described by Rabiner et al. (9), in which the high speed centrifugation was replaced by filtration through a series of micropore filters (105), observed a prolonged activated partial thromboplastin time (aPTT) in humans. Moss et al. (106) have shown that anticoagulant activity could be removed by treatment of their stroma-free hemoglobin solution with hydrated aluminum hydroxide gel at 37°C. Hedlund and Condie (67) observed that cardiac clot formation could be eliminated in dogs receiving their 7% stroma-free hemoglobin solution by first treating the hemoglobin solution with ion exchange resins to remove some of the non-hemoglobin contaminants.

Bolin et al. (15) have evaluated stroma-free hemoglobin solutions prepared by the ultrafiltration and crystallization methods (see section on hemoglobin purification) for their effect on various hematologic parameters in dogs, pigs and monkeys. Both types of solutions were shown to be free of contaminating lipids. The hematologic parameters studied included fibrinogen degradation products (FDP), aPTT, prothrombin time (PT), Kaolin clotting time (KCT), protamine sulfate precipitation (PSP), plasma complement, various plasma factors and platelet aggregation. These hemoglobin solutions produced no significant alterations in pigs and monkeys. In the dog, however, a consistent transient thrombocytopenia was observed and two of the ten dogs given hemoglobin solutions developed subclinical disseminated intravascular coagulopathy (DIC) as indicated by the FDP, positive PSP and a 70% drop in factor VIII. The nature of this species-specific response is not clear at the present, nor is its significance to humans (15). In in vitro tests on the effect of unmodified hemoglobin, pyridoxylated hemoglobin and pyridoxylated-polymerized hemoglobin on coagulation, only the unmodified hemoglobin showed procoagulant activity in the KCT test. In several other coagulation tests, including thrombin time (TT), aPTT and PT, no pro- or anticoagulant activity was demonstrated for the above hemoglobin solutions (21).

The absence of DIC in humans following infusion of autologous hemolysate (104) and the absence of coagulant activities by modified hemoglobin solutions (21) is encouraging to the development of hemoglobin-based blood substitutes. The observations of Savitsky et al. (14) and the species-specific hematological response induced in the dog by lipid-poor hemoglobin solutions (15), however, emphasize the need for careful evaluation of hemoglobin solutions for hematologic responses, both before clinical studies are undertaken and during the clinical evaluation.

The presence of vasoconstrictive activity has been demonstrated in hemolyzed erythrocytes (107-109), partially purified erythrocyte proteins (110), including non-hemoglobin fractions (111), and in hemoglobin and myoglobin solutions (112-115). Apstein et al. (112), using an in vitro isolated perfused rabbit heart assay, demonstrated a marked vasoconstrictive effect by stroma-free hemoglobin solutions compared to erythrocytes (116). Wellum et al. (114), studying the vasoactivity of various heme

proteins on canine basilar arteries, have shown that only proteins such as human and rabbit hemoglobins and horse-heart myoglobin, which have the iron in the ferrous oxidation state, were significantly vasoactive, whereas methemoglobin and cyanomethemoglobin showed very little vasoactivity. Bowman and Gillespie (113) showed that hemoglobin causes a powerful constriction of isolated bovine and canine penile arteries. Vasoactive effects induced by stroma-free hemoglobin solution have been demonstrated in humans by the appearance of bradycardia and mild hypertension (14). Martin *et al.* (115), studying the effect of heme proteins on the endothelium-dependent and glyceryl trinitrate induced relaxation of rabbit aorta, showed hemoglobin and myoglobin were effective inhibitors of the induced relaxation. They also observed that reduced cytochrome C was ineffective suggesting that only ferrous hemoproteins with ligand binding sites are inhibitory. The mechanism that has been suggested for the vasoactive effects of hemoglobin and myoglobin involves the inhibition of endothelium-derived relaxing factor(s) (EDRF) activity (115,117,118). This inhibition may involve the binding of EDRF by these heme proteins (115).

The presence of the vasoactivity in hemoglobin solutions requires that prospective hemoglobin-based blood substitutes are tested for vasoactive effects prior to clinical testing.

The effect of the hemoglobin's oxygen affinity on organ function has been demonstrated using isolated rabbit hearts. Perfusion of hearts with erythrocytes having low oxygen affinity delivered more oxygen to the heart and the myocardial oxygen consumption and contractile function were increased relative to the perfusion with erythrocytes having high oxygen affinity (119). Similarly, Benesch *et al.* (77) observed greater oxygen unloading and better mechanical performance in hearts which have been perfused with an interdimERICALLY crosslinked hemoglobin which has a low oxygen affinity, than with unmodified hemoglobin. In addition, the hearts perfused with the low oxygen affinity hemoglobin survived longer than those perfused with the unmodified hemoglobin. The above results illustrate the importance of the oxygen-affinity of hemoglobin solutions to the proper functioning of organs such as the heart, and that much better performance is obtained with low oxygen affinity hemoglobin.

The effects of hemoglobin solutions on the reticuloendothelial function have been discussed by DeVenuto in his 1979 review (18). Some of the early studies showed that hemoglobin solutions could block the reticuloendothelial system and make the animal more susceptible to infection. However, it was not clear whether the detrimental effects were caused by hemoglobin itself, or by contaminants such as the red blood cell stroma, in the hemoglobin solutions. More recent studies have shown that red blood cell stroma causes an acute depression of the reticuloendothelial system (RES) phagocytic function and an increased susceptibility to endotoxin shock (120,121). Hemoglobin injected at a dose of 45 mg/100 g of body weight, however, had no effect on RES function or on endotoxin shock susceptibility (122). Grover and Loegering (43) have shown that the increased susceptibility to streptococcus pneumoniae infection induced by red blood cell stroma was probably due to an impairment in hepatic clear-

ance and killing of the bacteria. The authors also suggested that the depressed bacterial clearance could have been contributed to by the stroma-induced decrease in complement levels. The above increased susceptibility to pneumococcal infection was observed with 0.5 mL/100 g dose of red blood cell stroma (112 ± 12 mg/mL Lowry protein and 96 ± 10 mg/mL hemoglobin), but not with a 0.3 mL/100 g dose. These observations suggest that the increased susceptibility to infection may be partially due to the overloading of the RES with stromal debris and by the depression in complement levels. These results, on the dependence of dose size, may also be relevant to studies on the effect of polymerized pyridoxylated hemoglobin on RES since the principle route of excretion of this high molecular weight hemoglobin is presumably the RES.

Greenburg (124) observed that if the animals are subjected to septic challenge 5 days after treatment with pyridoxylated hemoglobin, there was no difference in survival rates compared to controls and thus no depression in the animals' defence mechanisms at this time. Marks et al. (125) have studied the effect of polymerized pyridoxylated hemoglobin (SFHS-pp) (45 or 100 mg per 100 g of body weight) on the RES by observing the effect of SFHS-pp had on ^{131}I -gelatinized trioleate (GT) uptake by various organs and on the intravascular clearance rates of GT. Their results showed that there was no significant effect on the intravascular clearance rates 30 min after SFHS-pp administration. In addition, there was an overall stimulation of the reticuloendothelial function three days after SFHS-pp administration.

The above observations are encouraging in that the modified hemoglobin solutions do not seem to produce a depression of the RES function. It must be noted, however, that in the case of the pyridoxylated hemoglobin treatment, the animals were subjected to septic challenge five days after the hemoglobin treatment, and in the case of the polymerized pyridoxylated hemoglobin, the doses tested were relatively small compared to those that would be required during resuscitation. These experiments thus do not answer the question of the effect that a large dose of a hemoglobin solution would have on the RES function during the trauma period which is the period that the individual would be most susceptible to infection.

Dunn et al. (126) have shown that stroma-free hemoglobin is not toxic to polymorphonuclear neutrophils, although high concentrations of red blood cell stromal elements did diminish phagocytosis and bacterial killing by human neutrophils. The previous observations of an inhibitory effect on host neutrophil function and clearance of bacteria from the peritoneal cavity have been attributed to the presence of impurities and hemoglobin breakdown products in the commercial crystalline bovine hemoglobin that was used. The well-known infection potentiating effect of hemoglobin (127), however, has also been observed with the highly purified stroma-free hemoglobin (128). The mechanism of this adjuvant effect has recently been elucidated by the demonstration that bacteria growing in the presence of hemoglobin produce a leukotoxin thereby allowing the uncontrolled proliferation of the bacteria (129).

Greenburg (124) has addressed the question of iron handling by the

RES, however, it still remains to be shown what will be the effects of the large amount of iron that is added during a massive transfusion with hemoglobin solutions. The effects of stroma-free hemoglobin on the renal and hepatic systems have been discussed by DeVenuto (18). More recently, Friedman *et al.* (130) have shown that pyridoxylated-polymerized hemoglobin solutions produced no change in renal function as measured by blood urea nitrogen (BUN) and creatinine levels. In addition, animals receiving this hemoglobin solution displayed a normal hepatic morphology. These authors, however, have stated that additional studies are needed before this material can be recommended for clinical trials.

STORAGE AND STABILITY OF HEMOGLOBIN SOLUTIONS

Methods of storage and stability of hemoglobin solutions have been discussed by DeVenuto (18,21). Hemoglobin can be stored in a liquid or lyophilized form, cold or frozen, for up to two years without significant alteration in structure and function. Some of the more recent reports discuss stabilization procedures which include lyophilization and storage in the presence of stabilizers such as sucrose and amino acids (131,123). In addition, methemoglobin has been removed from hemoglobin solutions by reduction with sodium dithionite (44,115).

CONCLUSIONS

Polymerization of hemoglobin is currently the only means of obtaining a physiological concentration of hemoglobin which is iso-oncotic with plasma. Polymerized pyridoxylated hemoglobin is currently the most widely used example of such an iso-oncotic hemoglobin solution having normal oxygen carrying capacity. Such solutions, however, are heterogeneous polydispersed mixtures of widely different molecular weight. In addition, these solutions still contain about 20% of the unpolymerized tetramer which is rapidly eliminated in the urine. These solutions also have oxygen affinities which are considerably higher than those found in the red blood cells.

The polymerized pyridoxylated hemoglobin solutions have a plasma half-disappearance time of 25 hours in the rat (90) and 38 hours in baboons (24). In addition, they have been shown to support the life of rats which had 93-95% of their blood replaced, and baboons which received 900 ml of this solution (representing 50% of their blood volume) survived for over 40 months (1). It has, however, been shown that animals can survive normovolemic hemodilution with Dextran-70 to hematocrits as low as 5% (26,45). Thus, although it appears that these solutions are effective oxygen carriers, their efficacy must be confirmed in a variety of animal models that may include total exchange transfusion, resuscitation from normovolemic anemia and resuscitation from hemorrhagic shock (1).

In addition to their effectiveness, the hemoglobin solutions must be shown to be safe. There have been relatively few studies concerned with the safety aspects of the polymerized pyridoxylated hemoglobin solutions. Several areas of concern, however, have been identified in studies of stroma free hemoglobin solutions. Safety studies on the poly-

merized and modified hemoglobin solutions must address issues such as hematological responses, vasoactive effects, effect on the reticuloendothelial system, effect on immunocompetence, antigenicity, nephrotoxicity, and iron toxicity. In these studies, the range of hemoglobin concentrations should be chosen such that it covers the concentration that the individual would be exposed to during resuscitation. The composition of the hemoglobin solutions should also be defined as precisely as possible as the outcomes of the safety studies may be influenced by impurities present as well as by the nature of the hemoglobin.

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